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The hydrophobic surface of PaAMP from pokeweed seeds is essential to its interaction with fungal membrane lipids and the antifungal activity

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Abstract PaAMP is a small seed-specific antimicrobial protein from pokeweeds. It has a cysteine-knot fold with a positive patch and a hydrophobic surface. Site-specific mutagenesis was performed to study the roles of these two domains in antimicrobial activity and we found that the mutations in the hydrophobic surface had a more profound effect than that in the positive patch. A protein–membrane interaction was observed with the green fluorescence protein–PaAMP (GFP–AMP) fusion protein. The mutations that replace the amino acid residues forming hydrophobic surface with neutral residues abolished the interaction of PaAMP with the membrane and the binding of PaAMP to fungal sphingolipids while ergosterol enhanced the binding, suggesting that the hydrophobic surface was required for the interaction between PaAMP and fungal plasma membrane lipid raft.

Keywords: Antimicrobial protein; Pokeweeds; Site-specific mutagenesis; Sphingolipids

1. Introduction

Seed germination period in plant life cycle is probably most vulnerable time to microbial attacks because the rapture of seed coat would allow pathogen invasion into the storage tissue. It is thus not surprising that plant seeds produce a variety of antimicrobial proteins and peptides such as the plant defensins that have been isolated from seeds of many plants [1,2]. Another kind of antimicrobial protein has been reported in *Mirabilis jalapa* (MjAMP) and *Phytolacca americana* (PaAMP) [3–7]. PaAMP from pokeweeds is seed-specific and it is released from the germinating seeds to create an inhibitory zone against pathogens [5]. However, the mechanism of PaAMP against microorganisms is not understood at the moment.

The biochemical basis for the inhibition of yeast cells by the plant defensin DmAMP from *Dahlia merckii* has been studied and it is shown that sphingolipids are involved in DmAMP binding to the yeast plasma membranes [8,9]. The interaction between DmAMP and the yeast membrane could probably occur directly without the involvement of membrane proteins [9,10] and it probably leads to an insertion of the defensin into the membrane, resulting in an alteration of the membrane property and inhibition of fungal growth [11].

The structure of the PaAMP protein determined by NMR [12] has cysteine-knot folding with anti-parallel β-sheets stabilized by three disulfide bridges and it has a positive patch and a hydrophobic surface [5,12]. The two structural domains are so arranged to each other that the protein is strongly amphipathic. This feature of PaAMP is found in many antimicrobial proteins and it provides a hint that it might be involved in protein–membrane interaction. To study the mechanism of PaAMP against microorganisms and to understand the two domains in antimicrobial activity, site-specific mutagenesis was performed to specifically replace the residues in those two domains with neutral residues. We also constructed green fluorescence protein (GFP)–PaAMP fusion protein to locate the interaction site of the protein with fungal cells.

2. Materials and methods

2.1. Strains, Plasmids and site-specific mutagenesis

The *Escherichia coli* strain DH5α was used for all routine cloning purpose and the strain BL21(DE3)/pLysS was used for overproduction of recombinant proteins. The fungi *Fusarium solani* and *Neurospora crassa* were grown in YPG (0.3% yeast extract, 1% peptone, 2% glucose [pH 4.5]) medium at 30 °C.

The plasmids for overproduction of different PaAMP fusion proteins in *E. coli* were constructed as follows. The PaAMP gene was amplified by PCR with primers TTCCATGGCGGGATGCATAAAGAATGGGGG and TAATATAGTATGGGATCCTATCCCC using PaAMP cDNA as template. The amplified fragment was digested with *Nco*I and *Bam*HI and ligated into pET-30 to generate the plasmid pET30AMP for production of His-tagged PaAMP. To construct a plasmid overproducing GFP–PaAMP fusion protein, the PaAMP gene was amplified by PCR as above. The amplified fragment was digested with *Nco*I and *Bam*HI and ligated into pET-3d to generate the plasmid p3d-AMP. The modified GFP gene was amplified by PCR with the primers GGATCCATGGTGAGCAAGGGCGAGGAG and TTTGCCATGGCCTTGTACAGCTCGTCC using the plasmid pCOP1-GFP [13] as template. The amplified fragment was digested with *Nco*I before insertion into the *Nco*I site of p3d-AMP to generate the plasmid p3dGFP–AMP for overexpression of the translational fusion GFP–PaAMP gene. The construction of the PaAMP–GFP fusion gene and its overexpression will be described elsewhere.

Site-specific mutagenesis of the PaAMP gene was performed using the mutagenesis kit from Promega as described previously [14]. The following mutations were introduced into the PaAMP gene: K5A, N6R, F25A, I27A, V34A, K36E, R38N and I27A–V34A. These mutant genes were generated with primers: TACGGATCCGCGGGATGCATAGCGAATGG, TACGGATCCGCGGGATGCATAAAGAGAGG, TACGGATCCGCGGGATGCATAAAGGCAGG, ATTTGCGCGCAATAGCTAGAACAACAGTATG, ACACCATAGGATTGGCCGGCCGCTTGGAAACA, TAGGATTGTCCAG-

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CTATTTGGAAAC, TAAGAATTCTCAGCGGTTTTCGCAAACAC, and TAAGAATTCTCAGCGGTTTTCGCAAACAC, respectively. The mutant genes were confirmed by DNA sequencing. Overproduction of PaAMP and its derivatives were performed as described [15].

2.2. Protein purification

For purification of the His-tagged PaAMP overproduced in *E. coli*, the cell extracts were centrifuged at 30 000 × *g* for 40 min at 4 °C and the pellet was discarded. The PaAMP with His-tag was adsorbed to a Ni-chelating column (1.0 × 2.5 cm). The column was washed three times with phosphate butter before enterokinase was added to the column for cleavage of the fusion His-tagged PaAMP protein. The cleaved PaAMP was eluted from the column with the phosphate buffer and concentrated by ultrafiltration. For purification of both GFP–PaAMP and PaAMP–GFP fusion proteins, the cell extracts were centrifuged as above. To the supernatant, ammonium sulfate was added to a saturation of 60% and the solution was centrifuged again. The pellet containing the fusion proteins was resuspended in buffer A containing 20 mM HEPES (pH 7.0) and 10% ammonium sulfate was loaded onto a phenyl-Sepharose CL-4B (Amersham Pharmacia Biotech) column which has been equilibrated with buffer A. The fusion proteins were eluted with a gradient of ammonium sulfate from 10% to 0% and both fusion proteins were eluted nearly at the end of the gradient. The fusion proteins were further purified with a Sephadex G-50 filtration column (2.5 × 35 cm) and eluted with buffer A without ammonium sulfate at a flow rate of 15 mL h^{-1}.

2.3. Antimicrobial activity assays

The minimal inhibitory concentration of the proteins was determined according to Wu and Hancock [16]. Serial 2-fold dilutions of proteins were made in 20 mM Tris–HCl (pH 7.4) in a 96-well microtiter plate before mixing with 50 μl YPG broth in each well. To each well, 50 μl of YPG containing about 2×10^4 spores mL^{-1} were added. The plate was incubated at 25 °C for 48 h. The germination and growth of the spores were determined by measuring the culture absorbance at 595 nm using a microtiter plate reader (Bio-rad). The protein concentrations required for 50% growth inhibition (IC_{50}) after 48 h incubation were determined by plotting the growth inhibition versus protein concentrations [3]. A specific antimicrobial activity was defined as $1/IC_{50}$, and a relative activity of a protein was expressed as described by De Samblanx et al. [17]. For microscopic observation of the inhibition of spore germination or hypha growth of *F. solani*, the fungal spores or hyphae in 1 mL YPG containing PaAMP protein or its derivatives were incubated for 16 h at 25 °C with gentle shaking.

2.4. Other methods

Isolation of *N. crassa* sphingolipids and binding of PaAMP proteins to the sphingolipids were performed according to Thevissen et al. [10]. Protein concentration was determined by Lowry method [18]. Free cysteine thiol groups were determined by the Ellman assay on both reduced and unreduced proteins as described [19]. We found that less than 3% of the cysteine residues of the produced PaAMP proteins were in free cysteine form.

3. Results

3.1. The mutant PaAMP proteins and their antimicrobial activities

To study functions of individual amino acid residues of PaAMP by site-specific mutagenesis, it is necessary to isolate the protein in relatively large amount and to measure the activity of the mutant protein quickly. We thus established an overexpression system for PaAMP. We overproduced PaAMP as a fusion protein in *E. coli* and found that the fusion protein system of pET-30 was suitable for the purpose (Fig. 1). The fusion protein was produced in soluble form and we purified it with a metal affinity column. The purified fusion protein was then treated with enterokinase to remove the N-terminal part of the fusion protein. The expression system was also used to overproduce the mutant proteins of PaAMP constructed through site-specific mutagenesis (Table 1). We also constructed fusion genes encoding GFP–PaAMP, PaAMP–GFP and GFP–$PaAMP_{I26A–V34A}$ fusion protein. The overproduced fusion proteins were purified and analysed with SDS–PAGE (Fig. 1B).

The activity of rPaAMP against fungus *F. solani* was nearly identical to that of the PaAMP isolated from pokeweed seeds (Fig. 2A). The IC_{50} for PaAMP isolated from pokeweed seeds and rPaAMP against *F. solani* growth were 1.30 and 1.36 μM, respectively. This result suggests that the structure of rPaAMP is nearly identical to that of PaAMP present in pokeweed seeds. Fig. 2A also showed the inhibitory effects of both GFP–AMP and AMP–GFP. The values of IC_{50} and the relative activities of all rPaAMP proteins were summarized in Table 1.

The results of side-specific mutagenesis show that both the positive patch (including residues K36, R38 and K5) and the hydrophobic surface (including residues I27, F25 and V34) are important to the function of PaAMP. The K36A and R38A mutations resulted in a moderate 25% and 27% reduction of the antimicrobial activities, respectively (Table 1). A more profound effect was observed with $PaAMP_{K5A}$, which led to a 48% reduction of the antimicrobial activity. A similar result was reported for the defensin from radish seeds [17]. The mutation of N37R in PaAMP, which was constructed to increase the antimicrobial activity as in case of the V39R mutation of the radish defensin [17], reduced the activity by 50%.

Compared with the mutations in positive patch, all mutations in the hydrophobic surface had a more profound effect on the antimicrobial activity of PaAMP. The I27A, F25A and V34A mutations led to 72%, 81% and 82% reduction of the antimicrobial activity, respectively. The double mutation I27A–V34A led to a nearly complete loss of the antimicrobial activity. These results suggest that the hydrophobic surface of PaAMP is critical to the antimicrobial function of the protein.

PaAMP is inhibitory to the growth of another fungus *N. crassa* and the effects of mutations in both hydrophobic surface and positive domain were similar to that observed in *F. solani* (Fig. 2B). The values of IC_{50} for rPaAMP and $PaAMP_{K5A}$ on *N. crassa* were 1.93 and 4.59 μM, respectively. The PaAMP with double mutation of I27A and V34A ($PaAMP_{I–V}$) completely lost the antimicrobial activity against *N. crassa*.

3.2. Interaction of PaAMP with fungal membranes

The PaAMP–GFP fusion proteins are inhibitory to the growth *F. solani* and their activities were comparable to that of the rPaAMP (Table 1). Since GFP fusion protein could be observed with a fluorescence microscope, we used GFP–PaAMP fusion protein to localize the interaction site of PaAMP and the results are shown in Fig. 3. Addition of GFP–PaAMP fusion protein to the spore suspension at a concentration of 5 μM led to a complete inhibition of spore germination (Fig. 3A). Occasionally, binding of GFP–PaAMP to released protoplasm was observed (Fig. 3A2). Addition of GFP did not lead to any inhibition of the germination of the spores (Fig. 3B). No fluorescence could be observed from the germinating spores with GFP

(Fig. 3B2), suggesting that the PaAMP in the GFP–PaAMP fusion protein was required for both the interaction and the inhibition of fungal spores. The GFP–PaAMP fusion protein was also inhibitory to the growth of fungal hyphae. In the presence of GFP–PaAMP, the tips of fungal hyphae first showed ballooning (Fig. 3C1) and were green fluorescent (Fig. 3C2) before they burst and eventually release of all protoplasm with only empty walls left (Fig. 3C1). The fungal cell walls could not be labelled by GFP–PaAMP (Fig. 3C2), suggesting that they were not the interacting sites of PaAMP. The fusion protein of GFP–PaAMP with double mutation of I27A and V34A (GFP–PaAMP$_{I–V}$) and the fusion protein GFP–PaAMP with K5A mutation (GFP-PaAMP$_{K5A}$) were used for studying the roles of the hydrophobic surface and basic patch in interacting with fungal cells. GFP–PaAMP$_{I–V}$ did not inhibit the growth of the fungus and no labelling of the fungal hyphae by GFP–PaAMP$_{I–V}$ could be observed (Fig. 3D). This result strongly suggests that the hydrophobic surface of PaAMP is required for the interaction between the fungal membranes and PaAMP. Similarly, the spore germination of *F. solani* was not inhibited by GFP–PaAMP$_{I–V}$ (Fig. 3E1) and the cells were not labelled by GFP–PaAMP$_{I–V}$ (Fig. 3E2). Although, PaAMP$_{K5A}$ showed a nearly 50% reduction of its antimicrobial activity (Table 1), the GFP–PaAMP$_{K5A}$ protein can label the membranes of *F. solani* (Fig. 3F), suggesting that the basic patch of PaAMP plays a less important role in PaAMP–membrane interaction.

To further analyse the interaction of PaAMP and fungal membranes, we isolated sphingolipids from *N. crassa* and studied the binding of PaAMP with the lipids. As shown in Fig. 4A, rPaAMP had a dosage-dependent binding to sphingolipids. PaAMP$_{K5A}$could also bind to the sphingolipids. However, its binding efficiency was much lower than that of rPaAMP. PaAMP$_{I–V}$ showed little binding to the sphingolipids, confirming that the hydrophobic surface is critical to the interaction of PaAMP and the fungal lipids. The binding of PaAMP to sphingolipids was dependent upon the concentration

Table 1
The values of IC_{50} and relative activities of the recombinant PaAMP (rPaAMP) and its mutant proteins against *F. solani*

Proteins	IC_{50} (μM)	Relative activity
rPaAMP	1.36	100
PaAMP	1.30	104
N6R	2.57	53
K5A	2.72	50
K36E	1.90	71
R38A	1.91	70
F25A	6.96	19
I27A	4.90	28
V34A	7.21	18
I27A–V34A	17.68	7
GFP–AMP	2.31	60
AMP–GFP	2.20	67
GFP	>50	
Thioredoxin	>50	

Each IC_{50} value was determined as an average of three independent measurements as described in Fig. 2 and the relative activity is defined in Section 2.

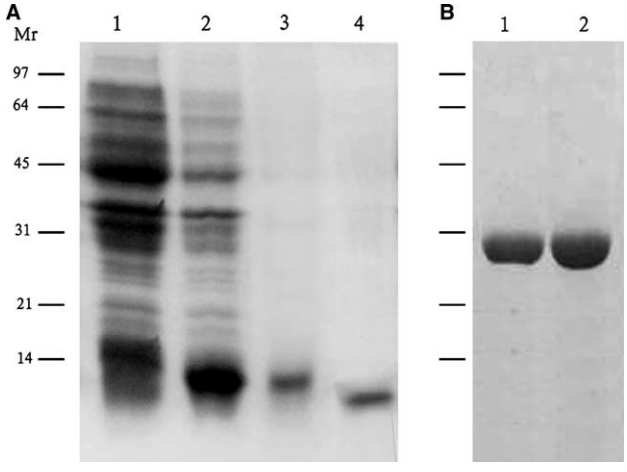

Fig. 1. Analyses of recombinant fusion PaAMP and their purification by SDS–PAGE. Panel A, Recombinant PaAMP (rPaAMP) was overproduced as a fusion protein with the expression vector pET30. Lane 1 and lane 2, whole cell extracts of *E. coli* cells containing pET30 and pET30AMP, respectively. Lane 3, rPaAMP purified with a metal-chelating column. Lane 4, rPaAMP after removal of the N-terminal fusion portion of the fusion protein. Panel B, purified GFP–AMP (lane 1) and AMP–GFP (lane 2) fusion proteins. The molecular mass markers are shown in the left side of the gels.

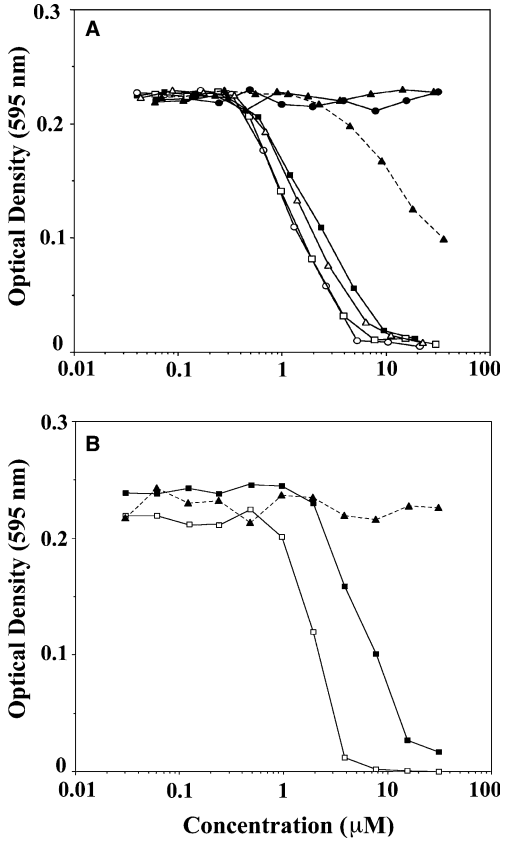

Fig. 2. Determination of values of IC_{50} of rPaAMP and its mutant derivatives. Recombinant PaAMP and mutant PaAMP proteins were first diluted in 2-fold series before they were mixed with the spore suspension of *F. solani* (A) or *N. crassa* (B). The growth of the spores were measured by monitoring the optical density at 595 nm after 24 h incubation at 25 °C in YPG medium. The values of optical density were plotted as functions of protein concentration (μM) in semilog plot. The effects of the following proteins were shown: GFP (—●—); thioredoxin (—▲—); PaAMP (—○—); rPaAMP (—□—); GFP–AMP (—Δ—); rPaAMP$_{K5A}$ (—■—); rPaAMP$_{I26A–V34A}$ (---▲---).

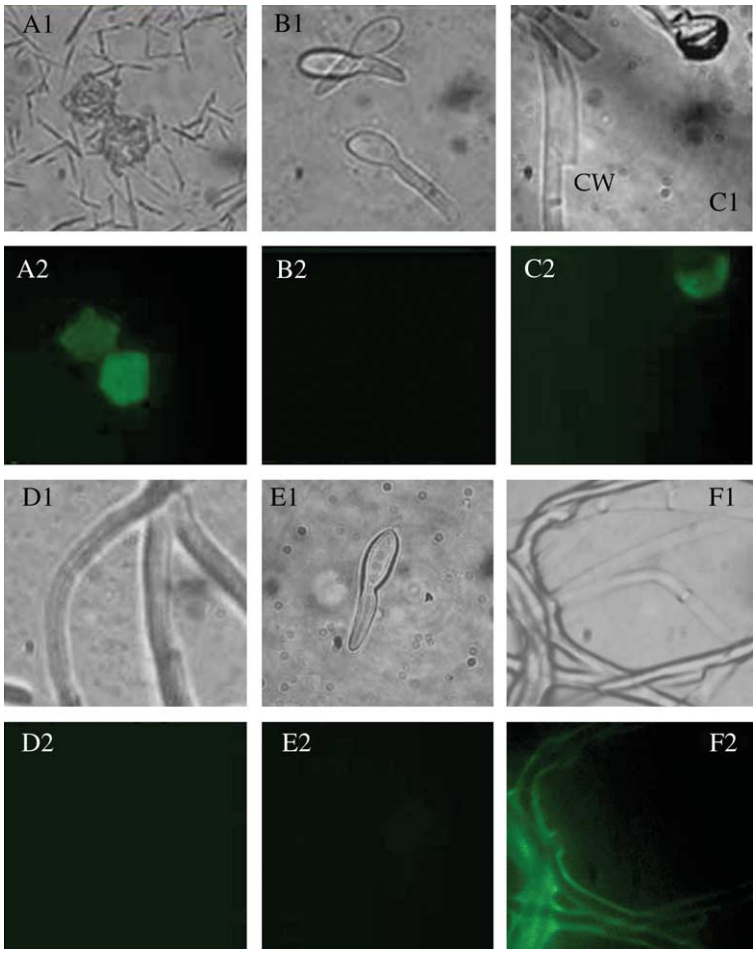

Fig. 3. Interaction of GFP–PaAMP fusion protein with the fungus *F. solani* observed with bright field microscopy (A1 through F1) or fluorescence microscopy (A2 through F2). (A) Incubation of the fungal spores with GFP–PaAMP inhibited their germination. The release of some protoplasm could be labelled by GFP–PaAMP fusion protein. (B) Spore germination in the presence of GFP. (C) Empty cell wall (CW) and ballooning of the hypha tip, which is green fluorescent, in the presence of GFP–PaAMP. (D) No inhibition of the growth of the fungal hyphae by GFP. The hypha protoplasm is not green fluorescent. (E) Germination of the spores in the presence of GFP–$PaAMP_{I26A–V34A}$. (F) Labelling of the fungus by GFP–$PaAMP_{K5A}$ as demonstrated by green fluorescent hyphae.

of sphingolipids (Fig. 4B). Under the experimental conditions, ≈130 pmol coated sphingolipids gave the highest binding of PaAMP. Ergosterol, which forms lipid raft with sphingolipids [20], enhanced the binding of PaAMP to sphingolipids (Fig. 4C), suggesting that lipid raft could be the site of PaAMP–membrane interaction. PaAMP did not bind ergosterol if the sphingolipids were not present (data not shown).

4. Discussion

The amphipathic feature of PaAMP is shared by many antimicrobial peptides and proteins and it plays an important role in antimicrobial activity [21]. In the present study, we show that overproduction of active PaAMP in *E. coli* can be obtained since the IC_{50} values for native and recombinant PaAMP on *F. solani* are nearly identical. Furthermore, the results based on site-specific mutagenesis of PaAMP show that the positive patch and the hydrophobic surface are both important for the antifungal function of PaAMP. Mutations in the hydrophobic surface, however, have a more profound effect on both antifungal activity and membrane-interacting properties. Mutations in the hydrophobic surface of PaAMP led to an inability of mutant PaAMP to interact with fungal plasma membranes while mutations in the positive patch did not disrupt the protein–membrane interaction (Fig. 3). The expression system used in this study is also suited for the overproduction of active GFP–PaAMP fusion proteins. The inhibition of fungal growth by both GFP–PaAMP and PaAMP–GFP (Table 1) suggests that PaAMP is folded as an independent structural domain in fusion proteins.

Since PaAMP is inhibitory to certain fungal species while no inhibitory effect of PaAMP is observed against plant cells, animal cells [5] and yeast species (*Saccharomyces cerevisiae* and *Pichia pastoris*, Peng and Zhao, unpublished data), the interaction of PaAMP and cell membranes must be specific [9]. Our results show that PaAMP interacts with sphingolipids from *N. crassa* and the interaction required the hydrophobic surface of PaAMP (Fig. 4). The binding of PaAMP to the sphingolipids is enhanced by ergosterol that forms lipid raft with sphingolipids [20], suggesting that lipid raft could be PaAMP's binding site as in case of DmAMP

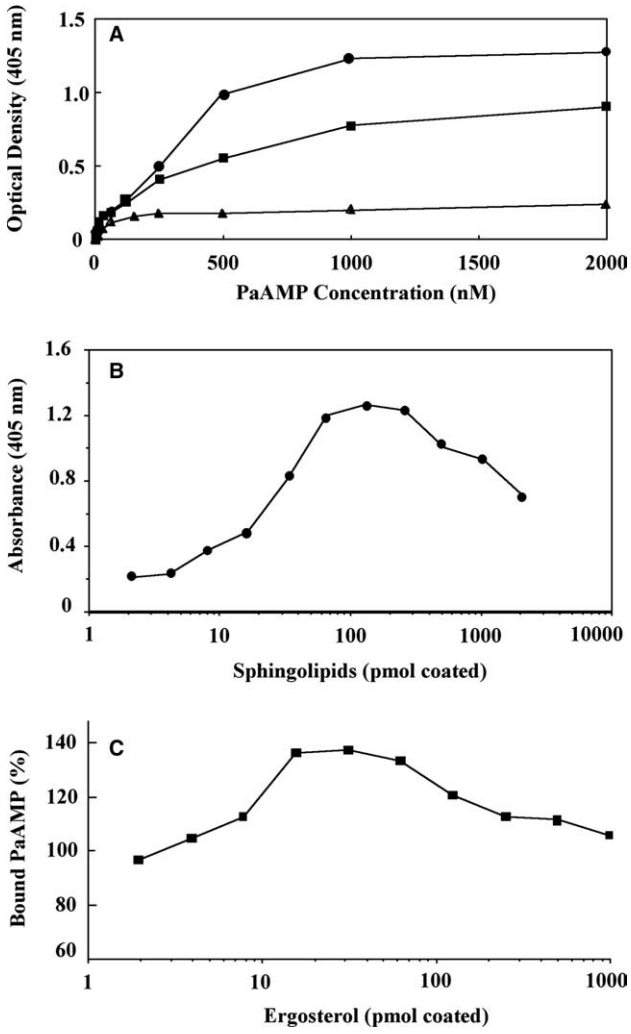

Fig. 4. Binding of PaAMP with sphingolipids from *N. crassa*. (A) Dosage-dependent binding of PaAMP proteins to the sphingolipids isolated from *N. crassa*. To each well of a microtiter plate coated with 130 pmol sphingolipids, PaAMP (circles), $PaAMP_{K5A}$ (squares) and $PaAMP_{I-V}$ at the concentrations indicated were added and the binding was determined with ELISA. (B) The dosage–response curve of the binding of PaAMP to sphingolipids. To wells of a microtiter plate coated with different amount of sphingolipids, 1.5 μM PaAMP was added and the binding was determined with ELISA. (C) Enhancement of PAMP binding to sphingolipids by ergosterol. Different amount of ergosterol was coated to the wells that contained 130 pmol sphingolipids and the binding of PaAMP was determined as in panel B. Data represent an average of triplicate measurements.

[9,10]. $PaAMP_{K5A}$ did not prevent PaAMP's binding to fungal membranes (Fig. 3), but it led to a reduced sphingolipid binding efficiency (Fig. 4). We speculate that mutations in the positive patch could lead to a reduced specificity of PaAMP–membrane lipid interaction, resulting in a decrease of antimicrobial activity.

Our results suggest that the antimicrobial mechanism of PaAMP is similar to that of DmAMP from *D. merckii*, which has been shown to interact with yeast sphingolipids [8–10], even though the structures of these two proteins are different. DmAMP is predicted to have a same structure of RaAFP2 [2] that is comprised of a βαββ fold stabilized by 4 disulfide bonds while PaAMP contains a cysteine-knot fold [5,12]. Further study is needed to know the exact nature of the fungal sphingolipids that specifically interact with PaAMP.

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